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Characterization of Type II Phosphatidylinositol 4-Kinase Isoforms Reveals Association of the Enzymes with Endosomal Vesicular Compartments*

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Phosphorylation of phosphatidylinositol (PI) to PI 4-phosphate is one of the key reactions in the production of phosphoinositides, lipid regulators of several cellular functions. This reaction is catalyzed by multiple enzymes that belong either to the type II or the type III family of PI 4-kinases. Type III enzymes are structurally similar to PI 3-kinases and are sensitive to PI 3-kinase inhibitors. In contrast, the recent cloning of the first type II PI 4-kinase enzyme defined a novel enzyme family. Here we characterize a new member of this family, the type IIβ enzyme that has been identified in the NCBI data base based on its homology to the first-cloned type IIα enzyme. The type IIβ enzyme has a primary transcript size of ~3.8 kb and shows wide tissue distribution. It contains an open reading frame of 1.4 kb, encoding a protein of ~54 kDa. Sequence comparison reveals a high degree of similarity to the type IIα enzyme within the C-terminal catalytic domain but significantly lower homology within the N-terminal region. Expression of both enzyme yields increased PI 4-kinase activity that is associated with the microsomal membrane fractions and is significantly lower for the type IIβ than the type IIα form. Both enzymes use PI as their primary substrate and have no detectable activity on PI monophosphates. Epitope-tagged as well as green fluorescent protein-tagged forms of both enzymes localize primarily to intracellular membranes and show prominent co-localization with early endosomes and recycling endosomes but not with the Golgi. These compartments participate in the processing of both the transferrin receptor and the G protein-coupled AT_{1A} angiotensin receptor. Our data indicate the existence of multiple forms of type II PI 4-kinase in mammalian cells and suggest that their functions are related to the endocytic pathway.

Inositol phospholipids have long been considered primarily as precursors for important messenger molecules during activation of certain G protein-coupled receptors and receptor-tyrosine kinases (1, 2). Phosphatidylinositol (PI)[1] 4-kinase and PI 4-phosphate (PI(4)P) 5-kinase activities were believed to maintain the small $PI(4,5)P_2$ pools of the plasma membrane during increased phospholipase C activity in stimulated cells. In addition to this important signaling aspect of phosphoinositide metabolism, it has been increasingly recognized in the last decade that localized phosphoinositide changes are of crucial importance in the organization of signaling microdomains (3, 4). A growing number of kinases and phosphatases that act upon inositides have been identified in recent years (5, 6). This together with the recognition and characterization of several molecular motifs that interact with inositides to regulate a large number of signaling molecules has contributed to our changing perception of how inositides contribute to cellular signaling. In light of such localized functions, the importance of PI(4)P formation has to be reevaluated. It can now be safely assumed that PI(4)P serves not only as a synthetic intermediate in $PI(4,5)P_2$ synthesis but also as a regulatory molecule on its own right.

PI 4-kinase (PI4K) enzyme activities have long been described in several tissues and have been classified as either type II or type III activities based on the catalytic properties of the enzyme (7–9). The sensitivity of type III but not type II enzymes to PI3K inhibitors (10) predicted a similarity between the catalytic domains of the type III enzymes and the PI3K enzymes. This has been proven by cloning of the type III PI4Ks (9, 11), of which two forms have been identified, a larger 200–220-kDa α-form and a smaller 110-kDa β-form, which are homologues of the yeast Stt4 and Pik1 proteins, respectively (9). Several elegant studies indicate that Pik1 and Stt4 serve nonredundant functions in yeast (12–15). Although Pik1 has been implicated in nuclear (12) and trans-Golgi (15, 16) functions, Stt4 was found to support cell wall synthesis and stability (15, 17). Most recently, it has also been shown that the PI(4)P pool produced by Stt4 but not by Pik1 is dephosphorylated by the inositide phosphatase, Sac1, and this lipid pool determines vacuole morphology and is functionally linked to the actin cytoskeleton (18). These studies are consistent with the existence of multiple functional pools of PI(4)P and tight control of their synthesis and degradation by distinct kinases and phosphatases.

In contrast to the significant progress in the field of type III PI4Ks, relatively little is known about the functions of the type II PI4Ks. Several biochemical studies demonstrate the presence of type II PI4K activity in a number of membrane compartments and organelles and indicate that the enzyme regulates PI(4)P synthesis related to several cellular processes, most notably to secretion (19). However, the molecular identi-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY065990.

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[1] The abbreviations used are: PI, phosphatidylinositol; PIP, phosphatidylinositol monophosphate; PI(4)P, PI 4-phosphate; $PI(4,5)P_2$, PI 4,5-bisphosphate; PI4K, PI 4-kinase; EEA1, early endosomal autoantigen; EGFP, enhanced green fluorescent protein (GFP); HA, hemagglutinin; PBS, phosphate-buffered saline.

ties of the type II enzymes have been only recently revealed. The enzyme has been cloned based on purification of the protein from secretory granules (20) and from detergent-insoluble membrane fractions, also termed rafts (21). The latter study also indicates the existence of homologues of the cloned enzyme identified in the EST data base and is termed the cloned enzyme type IIα, indicating that it was the first member of a family of enzymes.

In the present study, we have characterized the human type IIβ PI4K enzyme and compared its features to the human type IIα protein. We found significant differences in the tissue distribution and catalytic activities of the two proteins. We also demonstrate that both enzymes associate with the endosomal vesicular compartment in several cell types and is involved in the regulation of endosomal membrane traffic in mammalian cells.

EXPERIMENTAL PROCEDURES

Materials and DNA Clones—The EST clone from the I.M.A.G.E. Consortium (image.llnl.gov) (IMAGE clone ID 2905670, dbEST ID 5108611) encoding human PI4K type IIα was obtained from ATCC (Manassas, VA). The EST from Research Genetics (AL527283, dbEST ID 7860272) encoding human PI4K type IIβ was purchased from Invitrogen. The coding sequences of the two proteins were subcloned into the pcDNA3.1 plasmid for mammalian expression and *in vitro* translation or into the pEGFP-N1 plasmid to create the GFP-fused forms of the proteins. Epitope-tagged versions of the enzymes were created from the GFP fusion constructs by replacing the entire GFP sequence with the sequence coding for the HA epitope. To create a catalytically inactive enzyme, the conserved aspartate residue within the catalytic DRG sequence (Asp-308 in type IIα and Asp-304 in type IIβ) has been mutated to alanine by the QuikChange mutagenesis kit of Stratagene. To search for longer 5′ sequences, the type IIβ form was also amplified from Marathon Ready cDNA of human leukemia, K-562. Although longer forms have not been found, a shorter variant of the type IIβ enzyme has been isolated, and this was also subcloned into the pcDNA 3.1 and pEGFP-N1 plasmids. The TNT T7 Coupled Transcription/Translation System was obtained from Promega (Madison, WI). [γ-^{32}P]ATP (3000–6000 Ci/mmol) and [^{35}S]methionine were purchased from PerkinElmer Life Sciences. ATP, adenosine, and wortmannin were obtained from Sigma. Phosphatidylinositol was purchased from Fluka (Ronkonkoma, NY), and phosphatidylinositol phosphates were from Echelon Research Laboratories (Salt Lake City, UT). The primary antibodies against early endosomal autoantigen (EEA1) and gm130 were obtained from BD Biosciences. The Alexa-595 and Alexa-488 secondary antibodies were from Molecular Probes (Eugene, OR). The monoclonal anti-HA antibody was purchased from Covance, and the polyclonal anti-HA antibody was from Alpha Diagnostics (San Antonio, TX).

Northern Blot Analysis—Human 12-lane multiple tissue and cancer cell line Northern blots (CLONTECH) containing poly(A)$^+$-selected RNA were hybridized at 65 °C with the radiolabeled cDNA fragment using standard hybridization procedures (Amersham Biosciences). The 1.5-kbp *Eco*RI fragment containing the non-coding region of the type IIβ enzyme was used as a probe to detect the transcript for the type IIβ enzyme. For the type IIα enzyme, either a 500-bp PCR product coding for the unique N-terminal sequence or the full-length cDNA insert was used as a probe for hybridization. The cDNA fragments were labeled with the Prime-It RmT random primer labeling kit (Stratagene, La Jolla, CA). Membranes were washed twice for 15 min in 2× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate) with 0.1% SDS at room temperature followed by a 30-min wash in 0.1× SSC with 0.1% SDS at 60 °C.

In Vitro Translation—One microgram of supercoiled DNA plasmid was transcribed *in vitro* and then translated in the presence of [^{35}S]methionine with the TnT-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. The reaction products were analyzed by SDS-PAGE followed by autoradiography.

Immunocytochemistry and Confocal Microscopy—For immunostaining, COS-7 cells were grown on coverslips and fixed in 2% formaldehyde in PBS, pH 7.4, for 10 min at room temperature. After three washes with PBS (5 min each), fixed cells were incubated in blocking solution (10% FBS in PBS) for 10 min to decrease nonspecific binding of the antibodies. This blocking solution was complemented with 0.2% saponin for diluting the primary antibody (anti-EEA1 and anti-gm130, 1:250), and cells were incubated for 1 h at room temperature. After 3 washes, cells were incubated in the same buffer with a fluorescent secondary antibody (1:1000) for 1 h at room temperature. After a final washing step (3 × 5 min with blocking solution), the cells were rinsed with PBS, air-dried, and mounted on glass slides using Aqua Poly-Mount mounting medium (Polysciences, Inc.). Cells were then analyzed by confocal microscopy using an inverted Zeiss LSM-410 confocal microscope.

Immunoprecipitation of Epitope-tagged PI 4-Kinases—COS-7 cells cultured on 10-cm culture dishes were transfected with the respective HA-tagged PI 4-kinase constructs (or with GFP as control) for 48 h. Cells were lysed in 1 ml of lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin), and the lysates were cleared by centrifugation (14,000 × *g*, 15 min). After pre-clearing with 100 μl (1:1 slurry) of protein G-agarose for 30 min, 10 μg of monoclonal anti-HA antibody (MMS 101R, from Berkeley Antibody Co.) was added to the lysates, and the samples were incubated on a rotating platform at 4 °C for 2 h. The antibody was then collected on protein G-agarose beads (50 μl), and the complex was washed 3 times with 1 ml of lysis buffer before a final wash in the PI kinase buffer. The enzyme was then analyzed by Western blotting, or its activity was assayed on the beads as described below.

Assay of PI 4-Kinase—The activity of PI 4-kinase was measured as incorporation of radioactivity from [γ-^{32}P]ATP into organic solvent-extractable material as described previously (22). The standard reaction mixture for PI 4-kinase (50-μl final volume) contained 50 mM Tris/HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM EGTA, 1 mM PI, 0.4% Triton X-100, 0.5 mg/ml bovine serum albumin (lipid kinase buffer), 100 μM [γ-^{32}P]ATP, and the enzyme. All assay components except [γ-^{32}P]ATP were preincubated with or without inhibitors for 10 min at room temperature. Reactions were started by the addition of [γ-^{32}P]ATP and terminated after 10 min by the addition of 3 ml of $CHCl_3$, CH_3OH, 37% HCl (200:100:0.75 (v/v/v)). The organic solvent phase was separated from [γ-^{32}P]ATP as described elsewhere (10), and after evaporation, its activity was determined in a liquid scintillation counter. The identity of the lipid product was assessed by TLC analysis and by further phosphorylation with a recombinant type I PIP 5-kinase (kindly provided by Drs. Jolanta Vidugiriene and Thomas F. Martin).

The substrate specificity of the enzymes was measured with lipids spotted on nitrocellulose or SAM$^{2®}$ (Promega) membranes. 1–10 μg of lipid was spotted onto the membranes from a chloroform solution with or without phosphatidylserine. Dried membranes were incubated with the enzymes in the same buffer used for the kinase assays (except that it lacked PI, see above) in the presence of 100 μM [γ-^{32}P]ATP in a wet chamber for 1 h. Reactions were stopped with 50 mM EDTA, and the membranes were washed 3 times with 2 M NaCl followed by 3 washes in 2 M NaCl, 1% phosphoric acid and finally rinsed twice with distilled water. Phosphorylation of the lipids on the membrane was assessed by phosphorimaging analysis (PhosphorImager, Molecular Dynamics).

Permeabilized Cell Studies—COS-7 cells were seeded on 12-well plates (50,000 cells/well) and cultured for 2 days before transfection with LipofectAMINE 2000 according to the manufacturer's instructions. Twenty-four hours after transfection, cells were washed with PBS and incubated in 400 μl of permeabilization medium containing 110 mM KCl, 10 mM NaCl, 5 mM $MgCl_2$, 20 mM Hepes, pH 7.4, 2 mM EGTA, 0.05% bovine serum albumin, 15 μg/ml digitonin, 0.3 mM ATP, 12.5 μCi/ml [γ-^{32}P]ATP, and the various stimuli. Incubations were carried out at 37 °C for 10 min, and reactions were terminated with perchloric acid (5% final). Inositol lipids were extracted and separated by TLC as described previously (23), and their radioactivity was quantitated by phosphorimaging.

RESULTS

Molecular Characterization of Type IIβ PI4K—A search of the data base for homologues of the recently published type II PI4K revealed a protein sequence with significant homology with the type II PI4K enzyme (NCBI: 8922869). The nucleotide sequence for this polypeptide (XM003573) contained an overlapping segment with another nucleotide entry in the GenBank^{TM} (AK023186), providing a total transcript length of 3469 bp. An EST containing the full putative coding sequence (AL527283) was obtained, and its sequencing has confirmed the identity of the full cDNA sequence deduced from the two GenBank^{TM} sequences. This long transcript contains an open reading frame of 1503 bp (Fig. 1*A*). During our characterization

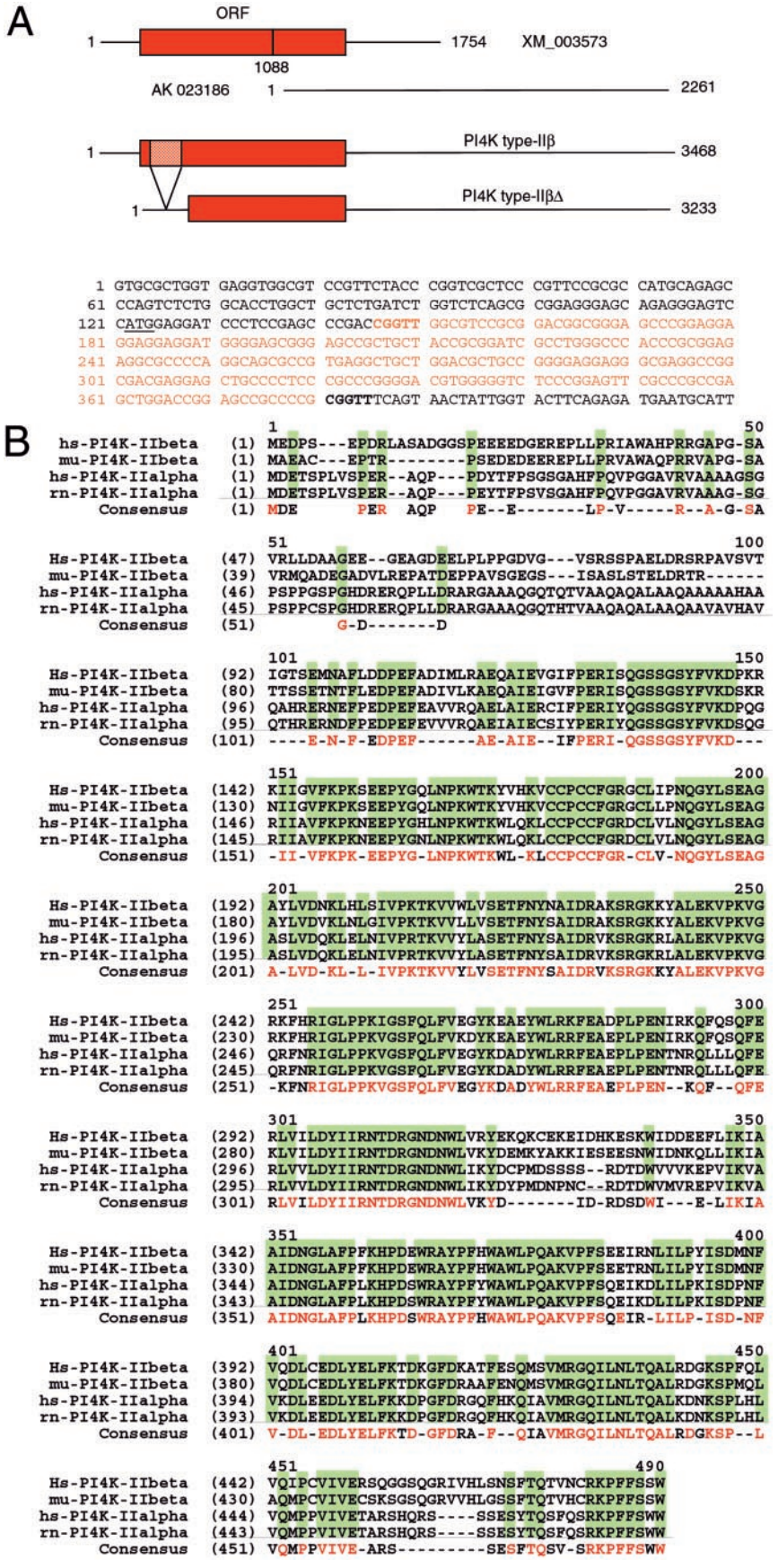

FIG. 1. **Structure and assembly of cDNAs encoding PI 4-kinase type IIβ (*A*) and amino acid sequence homology between the type IIβ and -α isoforms (*B*).** *Panel A*, The EST clone (AL527283) encompasses the two partial DNA entries in the GenBank™ encoding PI4K type IIβ. An alternative product obtained by PCR amplification from Marathon-Ready cDNA lacks the sequence labeled with *orange*, yielding an N-terminal-truncated from of the protein termed PI4K type II$\beta\Delta$. *ORF*, open reading frame. *Panel B*, sequence alignment of PI4K type IIβ and type IIα from human (*hs*), mouse (*mu*) and rat (*rn*). Conserved regions are *highlighted* with *green*.

of this sequence, Minogue *et al.* (21) reported the cloning of type IIα PI4K and also identified another protein sequence in the data base that was termed type IIβ and which is identical to the protein characterized in the present report. Therefore, we refer to this protein as the type IIβ PI 4-kinase. Fig. 1*B* shows the sequence homology between the type IIα and type IIβ enzymes.

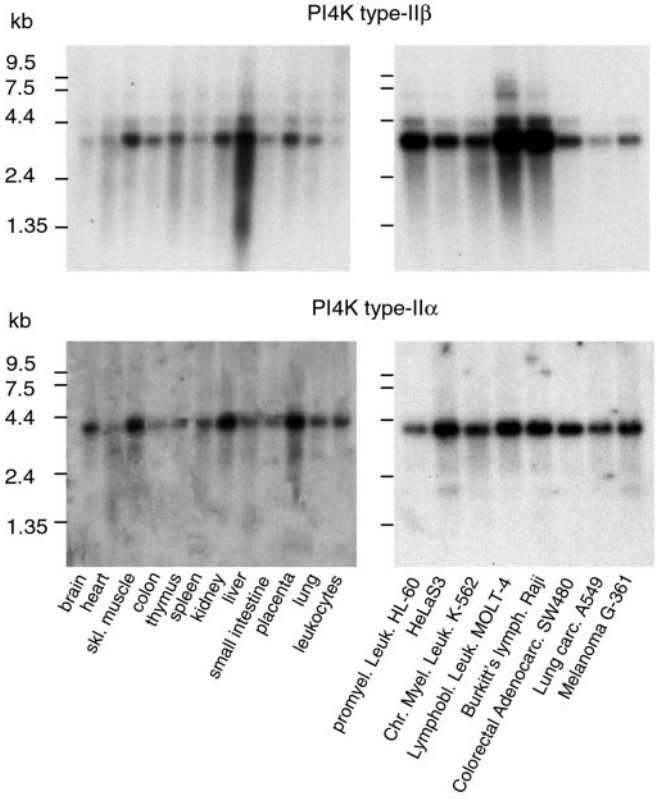

FIG. 2. **Northern analysis showing the distribution of PI4K type IIβ and PI4K type IIα mRNA in human tissues and cancer cell lines.** A human multiple-tissue Northern blot and a multiple cancer cell line Northern blot (CLONTECH) were hybridized with ^{32}P-labeled probes specific for the respective mRNAs. The exposure times for the type IIβ and type IIα blots were 16 h and 5 days, respectively. *skl.*, skeletal; *promyel. leuk.*, promyelocytic leukemia; *Chr.*, chronic; *Lymphobl.*, lymphoblastic; *lymph.*, lymphoma; *adenocarc.*, adenocarcinoma.

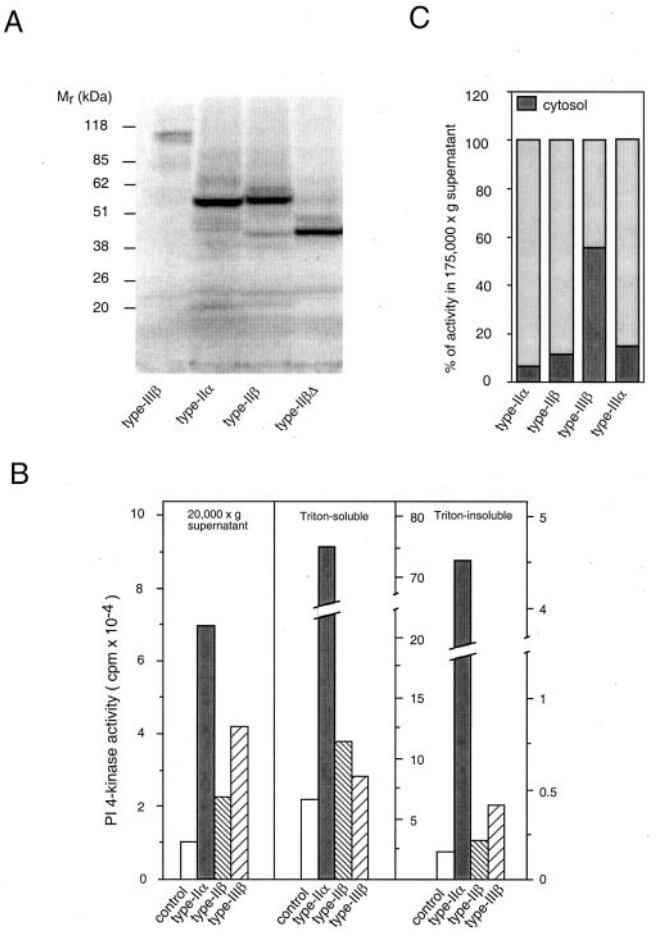

FIG. 3. ***In vitro*** **translation and expression of PI4K isoforms in COS-7 cells.** *Panel A*, cDNAs encoding PI4K type IIα, type IIβ, type IIβΔ, and type IIIβ were subcloned into the pcDNA3.1 mammalian expression plasmid and subjected to an *in vitro* translation reaction in the presence of [^{35}S]methionine using rabbit reticulocyte lysates. The reaction products were analyzed by SDS-PAGE. *Panel B*, COS-7 cells were transfected with the indicated plasmids (pEGFP was used as control), and after 24 h, cells were lysed, and their membranes were fractionated. PI 4-kinase activity was measured in the various fractions by an *in vitro* PI kinase assay. *Panel C*, the 20,000 × *g* supernatant was centrifuged with 175,000 × *g* to separate the light microsomal membranes from the cytosol, and the cytosolic activity was expressed as the percent of the total present in the 20,000 × *g* supernatant. Results from 3–4 representative experiments, each performed in duplicate, are shown; the error bars (less than 10%) are omitted for clarity.

A high degree of identity and similarity is found throughout the amino acid sequence, with only the N-terminal regions more unique. Because the transcript does not contain an in-frame stop codon preceding the putative translation start-site that conforms to a Kozak sequence, we searched for possible sequences extending in the 5′ direction using Marathon-Ready cDNA from various human tissues and cells. These efforts did not find any longer 5′ sequence but repeatedly identified a shorter transcript that lacks 235 bp in the N-terminal coding sequence, yielding a 96-amino acid shorter, N-terminal-truncated variant protein. This shorter form, which we termed type IIβΔ, could be an alternatively spliced form, although the lack of typical splice donor and acceptor sequences around the variant sequence (which lies within the first exon) makes this questionable. Given the sequence repeat around the "spliced-out sequence" in the short variant, it is possible that this is not a natural product but an artifact generated during cDNA synthesis (Fig. 1*A*). This question was not further pursued in the present study, but the short variant protein has proven to be useful to provide information about the role of the N-terminal sequence in the localization of the protein (see below).

Northern analysis was performed on human tissue mRNA blots using probes based on the non-coding region of the type IIβ and either the unique N-terminal or the full sequence of the type IIα enzyme. As shown in Fig. 2, a primary transcript size of ~3.8 kb was observed for both probes specific for the respective mRNAs and an additional weaker signal at ~4.3 kb in the case of type IIβ enzyme. Both transcripts showed a relatively uniform distribution between the tissues represented on the blots with only a few notable differences. These were the prominent abundance of type IIβ but not type IIα mRNA in liver and the relatively low level of type IIβ mRNA in the brain and peripheral leukocytes. A weaker signal was repeatedly observed with two distinct probes specific for the type IIα enzyme sequence. Probes based on the N-terminal short splice variant sequence of type IIβΔ failed to produce a detectable signal (not shown).

Biochemical Analysis of the Expressed Proteins—The coding sequences of the three proteins (type IIα, type IIβ, and type IIβΔ) were subcloned into the mammalian expression plasmid, pcDNA3.1. Proteins were first expressed in an *in vitro* translation reaction to reveal the sizes of the expressed proteins. As shown in Fig. 3*A*, type IIα, type IIβ, and type IIβΔ were all efficiently translated to yield proteins consistent with their expected molecular sizes. Importantly, the size of the *in vitro* translated type IIβ was the same regardless of the presence or absence of the large 3′-untranslated region, confirming the correct identification of the stop codon based on the nucleotide sequence. When the enzymes were expressed in COS-7 cells, a

large increase was observed in the PI4K activity of the cell lysates when cells expressed PI4K type IIα but only a moderate increase when the type IIβ protein was expressed (Fig. 3*B*). For a comparison, the two forms of the wortmannin-sensitive type III PI 4-kinases were also expressed in these studies. Most of the overexpressed type II activity was found to be membrane-associated and was solubilized with Triton X-100, as typically found for type II PI 4-kinases (Fig. 3*B*). However, some of the type II enzyme was also associated with the Triton-insoluble fraction and was also detectable in the 20,000 $\times$ *g* supernatant. In the latter fraction, however, most of the type II enzymes (unlike the type IIIβ form) was not cytosolic and was associated with the light membranes essentially as described in (20) (Fig. 3*C*). The effect of overexpression of the type II enzymes on the phosphorylation of endogenous PI was also examined in permeabilized COS-7 cells. Expression of the type IIα enzyme caused an average 2.5-fold increase in ^{32}P-labeling of PI(4)P, whereas the type IIβ enzyme caused only about a 20% increase, consistent with its significantly lower PI 4-kinase activity compared with that of α-form (Fig. 4*B*). Even the more active type IIα enzyme evoked only a moderate increase in the labeling of PI(4,5)P_2. This effect was more pronounced in the presence of 10 μM wortmannin, when the endogenous type III PI 4-kinases were inhibited (Fig. 4*A*).

To investigate whether the different activities of the α- and β-forms of the type II enzymes could be caused by their different optimum assay conditions, we examined the detergent sensitivities of the two enzymes. These experiments showed an identical activation of both enzymes with Triton X-100 in the same concentration range (not shown). When HA epitope-tagged forms of the enzymes were expressed in COS-7 cells and their expression levels were analyzed by Western blot analysis, a significantly lower level of expression of the type IIβ form was observed (Fig. 5*A*). Therefore, we performed immunoprecipitation and compared the activity of equal amounts of the two enzymes based on quantitation of the Western analysis. These measurements showed that the type IIβ enzyme was about 30% as active as the type IIα form (Fig. 5*B*). The reaction products of both enzymes were run together with PI(4)P on TLC analysis and could be converted to PI(4,5)P_2 by a recombinant type I PIP kinase, indicating that both enzymes are *bona fide* PI 4-kinases (Fig. 5*C*).

We also examined whether the type IIβ form can use alternative inositide substrates. However, neither enzyme could use any of the phosphorylated derivatives of PI as substrate *in vitro* under our experimental conditions (not shown). Comparison of the sensitivities of the two proteins to various inhibitors revealed their complete resistance to the PI 3-kinase inhibitor, wortmannin (not shown), and a slightly higher sensitivity of the type IIβ enzyme to phenylarsine oxide but not to adenosine (Fig. 6). It is worth noting that both enzymes were significantly more resistant to phenylarsine oxide than either of the two type III PI4K enzymes, as already reported for the type IIα enzyme (20). These data suggested that the lower PI 4-kinase activity of the β-form is not due to completely different catalytic properties or substrate specificity of the two enzymes.

Localization of the Type II Enzymes to Early Endosomes—To investigate the intracellular distribution of the two isoforms of type II PI4Ks, GFP-tagged as well as epitope-tagged versions of the proteins were created by fusing the enhanced GFP protein (or the HA epitope) to the C termini of the enzymes. These constructs were expressed in COS-7 and HEK 293 cells to observe the distribution of the expressed proteins. The GFP-tagged enzymes were catalytically active, but their activities were only about 50% of their untagged counterparts (data not shown). The cellular distribution of the proteins in live COS-7

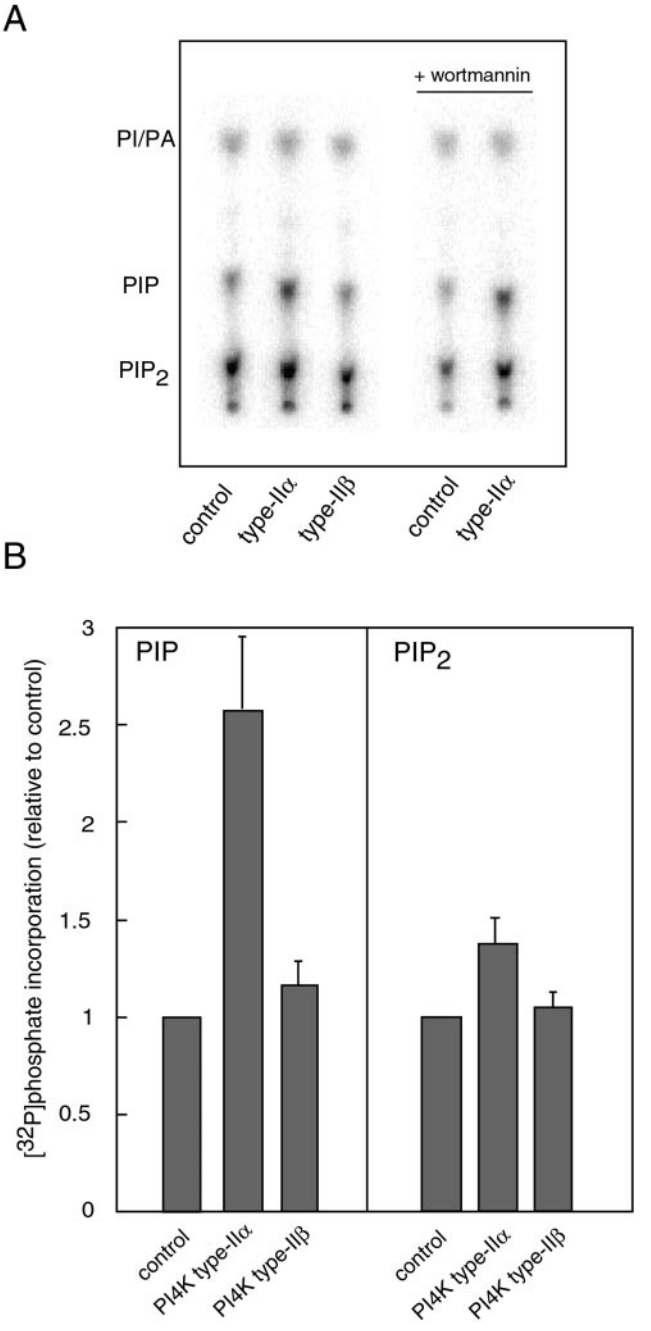

FIG. 4. **Effect of overexpression of PI4K type IIβ and type IIα on phosphorylation of endogenous lipid substrate in permeabilized COS-7 cells.** Cells were transfected with the indicated plasmids (pEGFP was used for control) for 24 h before analysis of [^{32}P]phosphate incorporation into various phospholipids from [γ-^{32}P]ATP after permeabilization with digitonin. *Panel A* shows the results of a representative TLC analysis, and *panel B* shows the summary of quantitative data from 4 similar experiments performed in duplicate. Wortmannin (10 μM) was added 10 min before permeabilization to inhibit endogenous type III PI 4-kinases. *PIP$_2$*, phosphatidylinositol biphosphate; *PA*, phosphatidic acid.

cells is shown in Fig. 7. Consistent with their tight membrane association, both type IIα and type IIβ forms were present in intracellular membranes, primarily in small vesicular structures scattered throughout the cytoplasm. Interestingly, the distribution and sizes of the vesicles positive for the type IIα enzyme were clearly dependent on the level of protein expression. Cells that expressed high levels of the protein contained larger vesicles that were concentrated mostly in the juxtanuclear compartment (Fig. 7, *A–C*). Higher expression levels

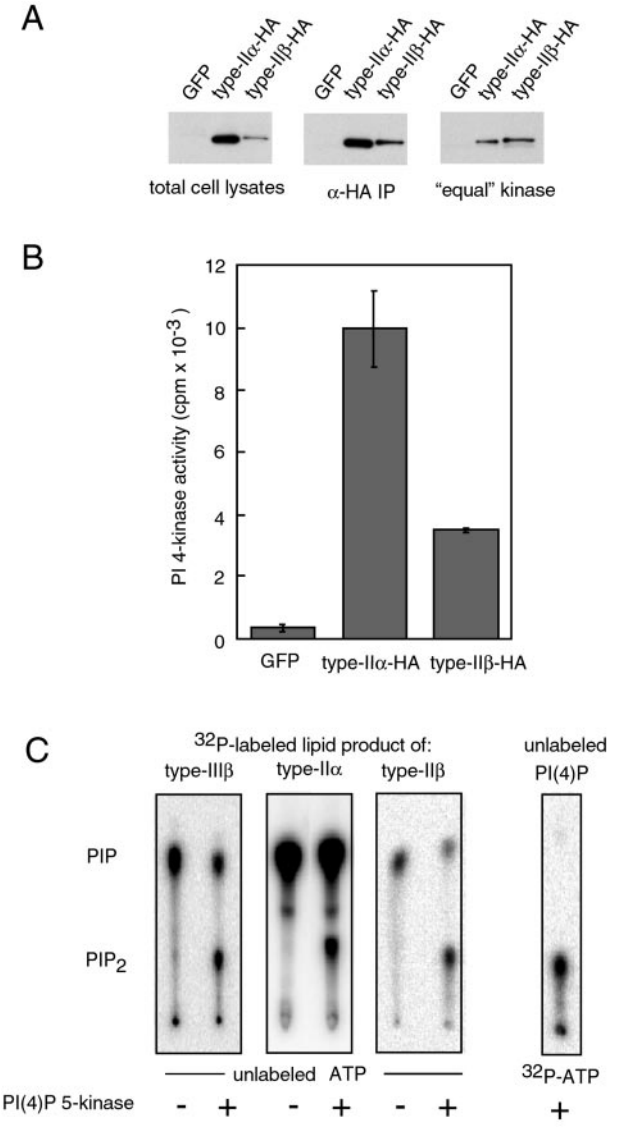

FIG. 5. **Comparison of expression levels and activities of epitope-tagged PI4K type II enzymes.** COS-7 cells were transfected with either PI4K type IIα or type IIβ, epitope-tagged at their C termini with the HA epitope. Total cell lysates as well as the immunoprecipitated (with a monoclonal anti HA antibody) proteins were analyzed by Western blotting using a polyclonal anti HA antibody (*panel A*). Based on densitometry, "equal" amounts of the two kinases were assayed for PI kinase activity (*panel B*) and again analyzed by Western blotting (*right* on *panel A*). The identity of the lipid product was determined by TLC analysis and further phosphorylation by a type I PIP 5-kinase, which converts PI(4)P but not PI(5)P to PI(4,5)P_2 (*panel C*).

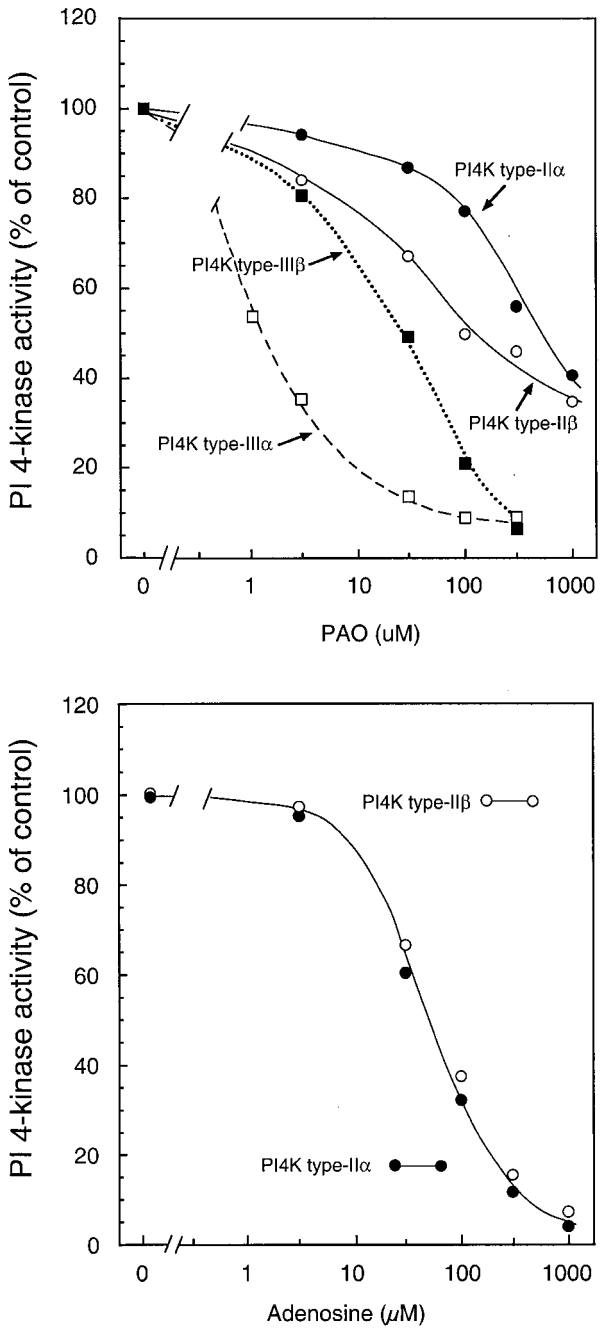

FIG. 6. **Sensitivity of the individual PI4K isoforms to phenylarsine oxide (*PAO*) and adenosine.** COS-7 cells were transfected with the indicated plasmids or pEGFP for 24 h. After lysis and fractionation (see the legend to Fig. 3), PI 4-kinase activities of the 20,000 × *g* supernatant (for the type III enzymes) or of the Triton X-100-sulubilized membranes (for the type II enzymes) were assayed after a 10-min preincubation with the indicated concentrations of inhibitors. In each case, the activity of the pEGFP-transfected control assayed under similar conditions was subtracted, and the results are expressed as the percent of the activity measured without inhibitors. The average results from two experiments are shown, and the error bars (less than 10%) are omitted for clarity.

of the type IIβ protein also caused the appearance of larger vesicles, but juxtanuclear accumulation of these enlarged vesicles was not as obvious as that of the type IIα enzyme (Fig. 7, *D–F*). Plasma membrane localization was less pronounced in the case of the type IIβ enzyme, and more of this protein was present in the cytoplasm (Fig. 7*D*). The shorter, type II$\beta\Delta$ enzyme, on the other hand, failed to show membrane localization and was mostly present in the cytoplasm (Fig 7, *G–I*). This result indicated that the N-terminal 96-amino acid sequence is necessary to target the protein to its specific membrane location. Immuno-cytochemical analysis of the epitope-tagged enzymes in fixed cells showed a distribution that was indistinguishable from that of the GFP-fused forms. Moreover, simultaneous detection of the GFP-tagged and epitope-tagged versions of the same expressed enzymes showed clear co-localization for both the type IIα and type IIβ enzymes (data not shown). To determine the identity of the membrane compartment in which the enzymes were found, transfected COS-7 cells were fixed and subjected to immuno-cytochemistry using antibodies against known intracellular markers. These studies showed that both the α and β forms of the enzymes co-localized with the EEA1 protein in the small peripheral membrane vesicles, suggesting their association with early endosomes (Figs. 8 and 9). Similar data were obtained with the epitope-tagged

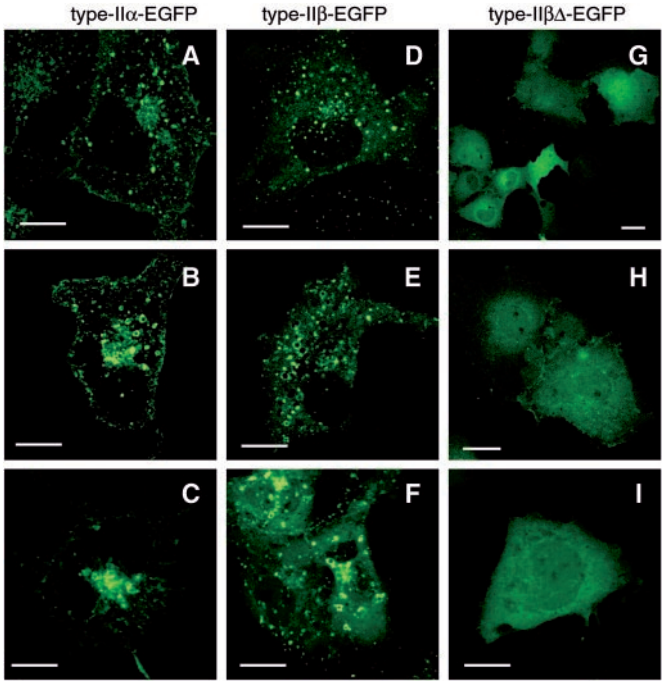

FIG. 7. **Cellular distribution of type II PI 4-kinase-EGFP isoforms, expressed in COS-7 cells.** EGFP was fused to the C termini of the two PI4K type II isoforms, and the hybrids were expressed in COS-7 cells. One day after transfection, live cells were analyzed in an inverted Zeiss LSM-410 confocal microscope. Cells expressing increasing amounts of the kinase (*panels A–C* and *D–F* from *top* to *bottom*) show larger vesicles (*B* and *E*) and, in the case of the type IIα enzyme, also show accumulation of the larger vesicles in the juxtanuclear compartment (*C*). Association of the type IIβ kinase with the intracellular vesicles requires the N-terminal 96 amino acids, since the truncated enzyme is largely cytosolic (*panels G–I*). The *bars* represent 10 μm.

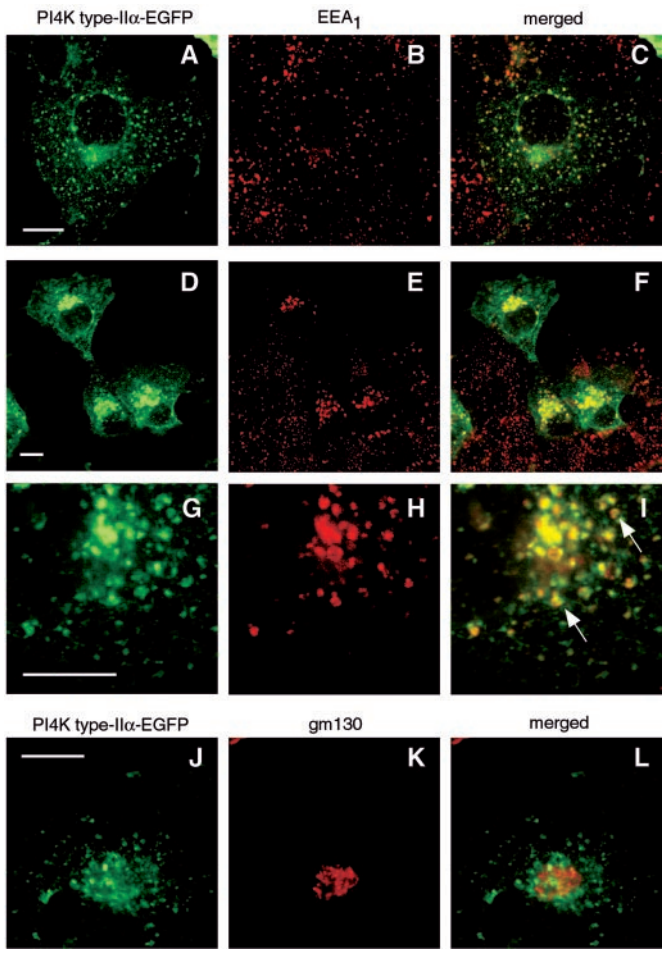

FIG. 8. **Co-localization of the type IIα PI4K with the early endosome-associated autoantigen (EEA1).** COS-7 cells expressing PI4K type IIα fused to EGFP were fixed and permeabilized for immunocytochemical analysis using, EEA1 (*A–I*) and the Golgi marker, gm130 (*J–L*). Co-localization of the type IIα enzyme with EEA1 in the small punctate structures scattered around the cytoplasm is clearly evident (*A–C*). At higher expression levels, cells contain larger vesicles that are also positive for EEA1 (*D–F*). It is noteworthy that the *red* and *green* signals do not exactly overlap within the same vesicular structures, as if their distribution had some polarity (see *arrow* in *panel I*). No co-localization of the kinase is observed with the Golgi marker, gm130 (*J–L*). The *bar* represents 10 μm.

enzymes (not shown). In cells expressing high levels of the type IIα or type IIβ enzyme, the enlarged vesicles were also positive for the EEA1 protein. In contrast, no co-localization of the type IIα PI4K enzyme was observed with the Golgi marker protein, gm130, even in cells where the type IIα enzyme was found in the juxtanuclear vesicular compartment (Figs. 8 and 9). In the case of the type IIβ form, some cells showed a signal over the area of the Golgi (this was more prominent in the fixed cells), but the majority of the signal was associated with the vesicular endosomal structures (Fig. 9).

Association of Type II PI 4-Kinases with the Endocytic Pathway That Processes Both Transferrin and G Protein-coupled Receptors—To investigate whether the type II enzymes are present on the endocytic pathway through which internalized cell surface receptors are processed, we examined the uptake of Alexa-594-conjugated transferrin in COS-7 cells expressing the GFP-tagged forms of the respective type II enzymes. As shown in Fig. 10, co-localization of transferrin with either the type IIα or type IIβ enzyme was clearly demonstrable in early endosomes during endocytosis of the fluorescent ligand. At later times (>15 min), when transferrin began to accumulate in juxtanuclear recycling endosomes, it showed co-localization with the type IIα enzyme present in this compartment in cells expressing higher levels of the enzyme. The presence of high levels of the type IIα enzyme reduced the uptake of transferrin compared with non-transfected cells (Fig. 10), indicating that accumulation of vesicles in the juxtanuclear recycling compartment is probably associated with reduced recycling of transferrin receptors to the plasma membrane. A similar inhibitory effect of the type IIβ enzyme on transferrin uptake was not appreciable.

When catalytically inactive mutant forms of the enzymes were expressed in COS-7 cells, their distribution showed subtle differences compared with their wild-type counterparts. These included a more prominent plasma membrane localization of the inactive type IIα form and the accumulation of numerous vesicles in the juxtanuclear region of the cell (Fig. 11*A*). In addition, small tubular structures were observed in some of the cells expressing high levels of the kinase-inactive proteins, and these were much more pronounced in the case of the inactive type IIβ enzyme (Fig. 11*B*). Unlike its wild-type form, kinase-inactive type IIβ did inhibit transferrin uptake (Fig. 11*B*). Nevertheless, transferrin uptake was observed in many cells expressing lower levels of the proteins after prolonged incubations (not shown). Co-localization of the GFP-tagged type IIα enzyme with G protein-coupled receptors was also examined in HEK 293 cells stably expressing the AT_{1A} angiotensin receptor. As shown in Fig. 12, after stimulation with rhodamine-conjugated angiotensin II, the ligand appeared in the vesicular compartments that were positive for type II PI4K, indicating that AT_{1A} receptors are also sorted through these PI4K-positive vesicles during agonist-induced endocytosis.

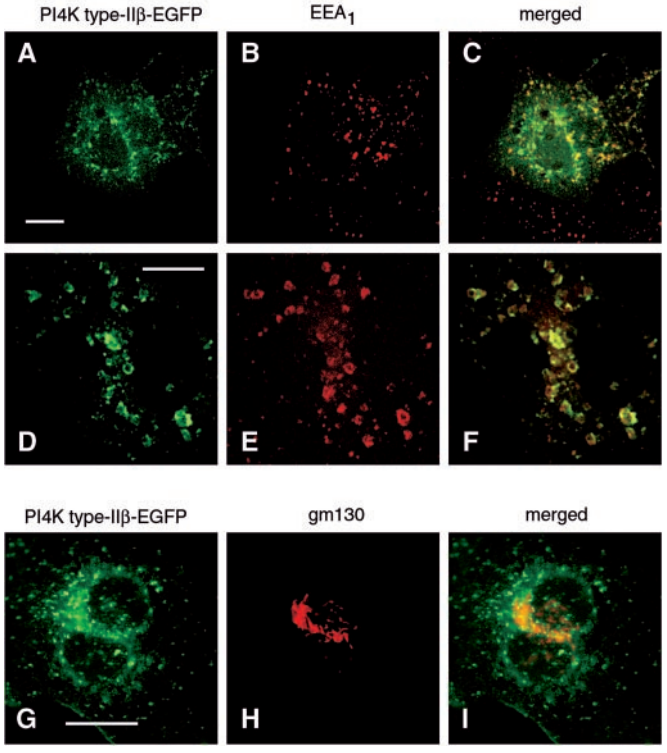

FIG. 9. **Co-localization of the type IIβ PI4K with the EEA1.** COS-7 cells expressing PI4K type IIβ fused to EGFP were fixed and permeabilized for immunocytochemical analysis using the early endosomal marker, EEA1 (*A–F*), and the Golgi marker, gm130 (*G–I*). Like type IIα, type IIβ is co-localized with EEA1 in the small punctate structures scattered around the cytoplasm (*A–C*) and in the larger vesicles that can be observed in cells expressing the kinase at higher levels (*D–F*). Again, no co-localization of the kinase is observed with the Golgi marker, gm130 (*G–I*). The *bar* represents 10 μm.

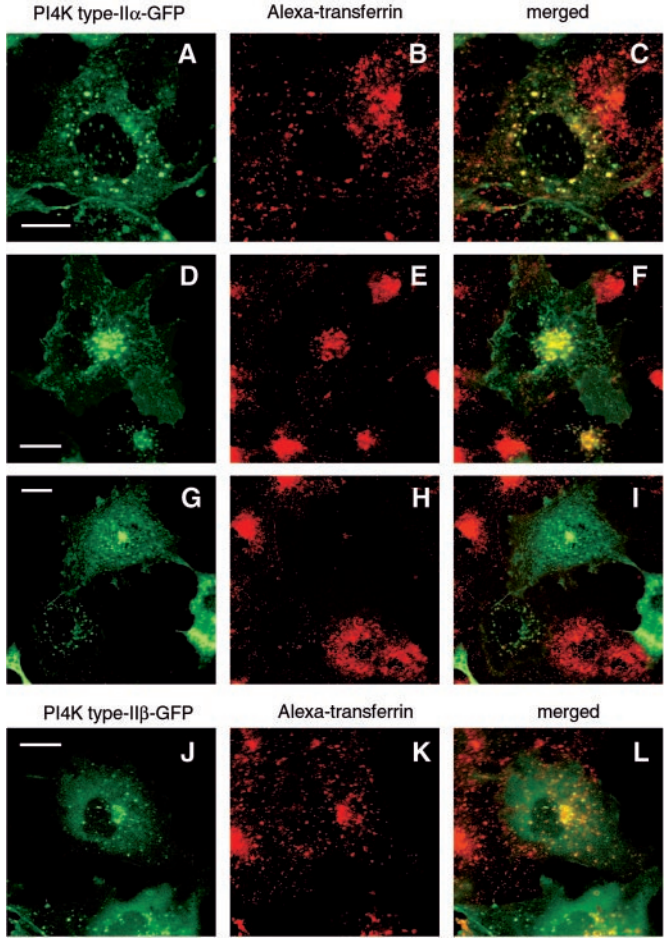

FIG. 10. **Co-localization of type II PI4K isoforms with Alexa transferrin in COS-7 cells.** COS-7 cells expressing PI4K type IIα (*A–I*) or type IIβ (*J–L*), both fused to EGFP, were incubated at 33 °C with Alexa-594-labeled transferrin for increasing periods of time. After incubation for 5–15 min, co-localization of Alexa transferrin with both the α and β forms of the kinase is observed over the early endosomes (*A–C* and *J–L*, respectively). At later times (20–30 min), transferrin also appears in the juxtanuclear recycling endosomes, where it also co-localizes with vesicles containing the type IIα kinase (*D–F*). Most of these structures are positive for the presence of the kinase in cells expressing high levels of the type IIα PI4K. Loading of the recycling endosomes with Alexa transferrin is greatly reduced in cells that express moderate to high levels of PI4K type IIα (*G–I*). The *bar* represents 10 μm.

DISCUSSION

Type II PI 4-kinase was the first PI kinase to be biochemically characterized and purified from several membrane sources, including red blood cell membranes, liver, bovine uterus, and A431 cell membranes and also from *Saccharomyces cerevisiae* (8, 9). This tightly membrane-bound enzyme is responsible for the majority of the PI 4-kinase activity found in the membranes of mammalian cells. Type II PI 4-kinases have been distinguished from other PI 4-kinases by their sensitivity to low concentrations of adenosine (K_i 10–50 μM) and micromolar concentrations of Ca^{2+} as well as to the anti-type II PI 4-kinase-neutralizing antibody, 4C5G (7). Based on these criteria, type II PI 4-kinases have been shown to be associated with virtually every membrane compartment within the cell including the plasma membrane, Golgi, secretory vesicles, and lysosomes in studies using cell or tissue fractionation (8, 9). However, the regulatory roles of these enzymes within these or any other compartments have not yet been clearly defined.

Despite their wide tissue distribution and prominent activity, the molecular identity of type II PI 4-kinases remained elusive until very recently, when two groups independently cloned the enzyme after purification of the protein from the membranes of chromaffin granules (20) and from non-caveolar membrane rafts, a subdomain of the plasma membrane (21). The reported enzymatic properties of the cloned protein are clearly consistent with it being a type II PI 4-kinase. Sequence homologues of type II PI 4-kinases have been identified in other species including *S. cerevisiae* in the NCBI data base. PI4K type IIβ, a closely related protein already noted in Minogue *et al.* (21) and characterized in this report, displayed a weaker PI 4-kinase activity than the type IIα enzyme, even after correction for its lower expression levels. Nevertheless, despite their remarkably different PI kinase activities, these two proteins have similar catalytic properties, inhibitor sensitivities, and substrate specificities. This raises the possibility that some additional members of this enzyme family may not even possess PI kinase activity and could be protein kinases similarly to the members of the PI 3-kinase-related kinases (24). It is noteworthy that the yeast homologues of the two type III PI 4-kinases, Pik1p and Stt4p, account for more than 90% of the yeast PI 4-kinase activity (15), raising the question of whether the yeast homologue of the type II PI 4-kinase possesses significant PI kinase activity. Whether any of the type II enzymes display protein kinase activity has yet to be determined, but among the possible inositide lipid substrates, these enzymes can only phosphorylate PI.

The intracellular localization of the two PI 4-kinase isoforms showed significant similarities and only subtle differences. Both enzymes were found to be associated with intracellular vesicular membranes bearing the early endosome marker, EEA1, and in some cells with the juxtanuclear recycling endo-

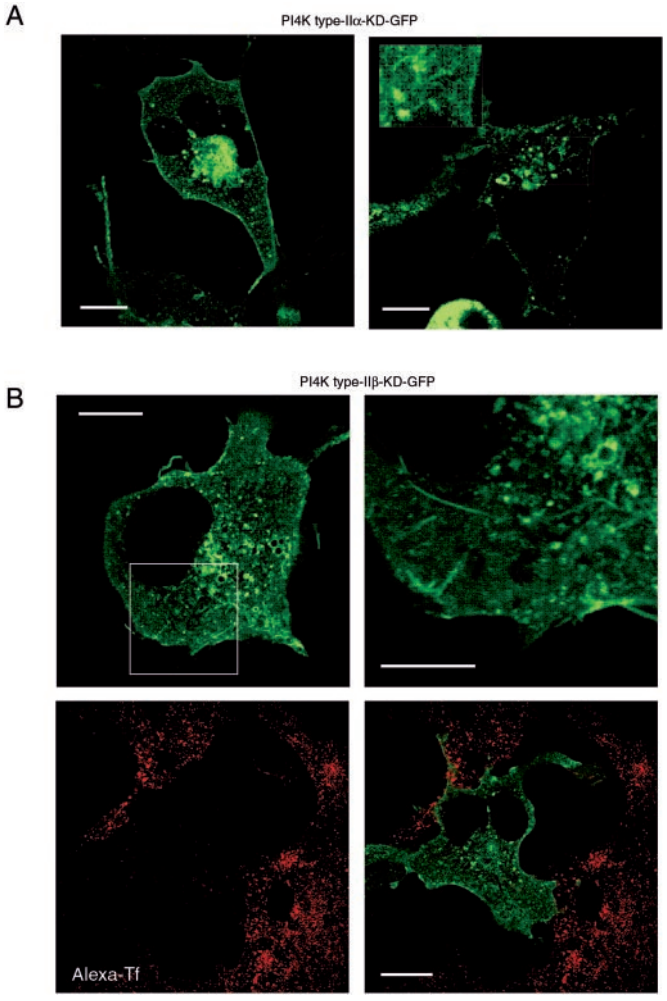

FIG. 11. **Cellular distribution of kinase-inactive type II PI 4-kinase isoforms, expressed as GFP fusion proteins in COS-7 cells.** COS-7 cells were transfected with the kinase-inactive mutants of the respective enzymes (D308A of type IIα, *panel A*, and D304A of type IIβ, *panel B*). Note the intense plasma membrane localization of the enzyme and the accumulation of juxtanuclear vesicles in *panel A*. The *inset* shows tubular structures that can be observed beneath the plasma membrane. *Panel B*, the tubular structures are more prominent with the kinase-inactive PI4K type IIβ enzyme. Also, the uptake of Alexa transferrin (*red*) (5-min pulse and 5-min chase at 37 °C) is greatly reduced in cells expressing high amounts of kinase-inactive PI4K type IIβ. The *bars* represent 10 μm.

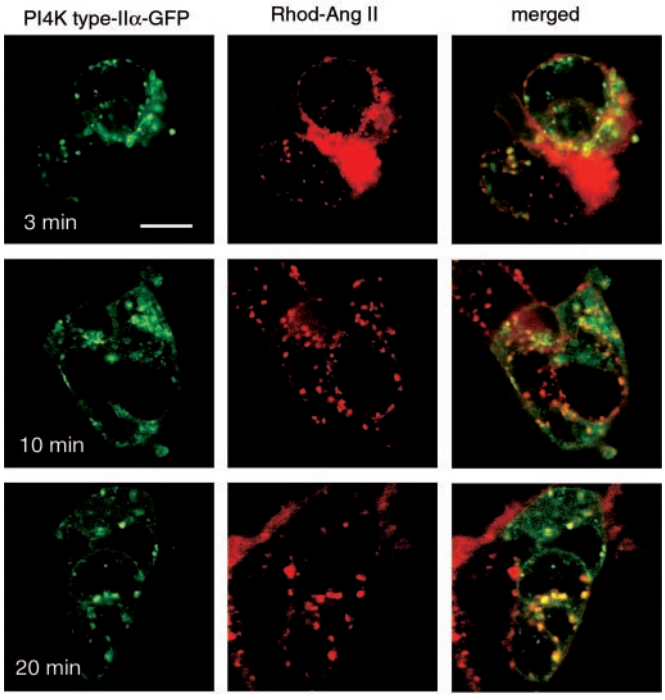

FIG. 12. **Co-localization of type II PI4K isoforms with internalized rhodamine-angiotensin II in HEK-293 cells stably transfected with the AT_{1A} angiotensin receptor.** HEK-293 cells expressing the AT_{1A} angiotensin receptor were transfected with PI4K type IIα fused to EGFP. One day later, cells were incubated in the presence of rhodamine-labeled angiotensin II (*Rhod-Ang II*) at 33 °C for the indicated periods of time. Co-localization of the ligand (*red*) and the kinase (*green*) in early endosomes is evident shortly after stimulation. The *bar* represents 10 μm.

somes. The expressed type IIα enzyme fused to EGFP also clearly promoted the formation of recycling endosomes, since this compartment was prominently present in cells expressing high levels of the protein. This effect was not pronounced with the type IIβ enzyme, perhaps due to its lower PI 4-kinase activity. Association of both type II PI 4-kinases with the endosomal vesicular pathway carrying both internalized transferrin as well as the ligand of the G protein-coupled AT_1 angiotensin receptor was clearly demonstrable. This finding indicates that type II PI 4-kinase(s) may participate in the trafficking steps associated with clathrin-mediated endocytosis. Although the roles of Class III and Class II PI 3-kinases have been well documented in the endocytic process (25, 26), PI 4-kinases have not yet been implicated despite the known requirement for $PI(4,5)P_2$ binding to several proteins that participate in clathrin assembly (27, 28). A recent study has shown that plasma membrane removal and recycling is greatly affected by both ARF6 and the type I PIP 5-kinase (29). Because PIP 5-kinase uses PI(4)P as its substrate, type II PI 4-kinases are good candidates for producing PI(4)P in these internalized membranes, especially since none of the type III PI 4-kinases appear to be present in these cellular compartments (30). The reported association of the type II PI 4-kinase activity with the epidermal growth factor receptor after agonist stimulation (31, 32) could also be related to the endocytosis and subsequent processing of this receptor.

Expression of kinase-inactive mutants of both proteins exerted no prominent change in cellular morphology other than what has already been observed with the kinase active forms, which is the formation of larger vesicles that often accumulated in the juxtanuclear compartment. The only clear effect of overexpressed kinase-inactive enzymes was the appearance of fine tubular structures of variable length at the cell periphery, and this effect was significantly more pronounced with the type IIβ form. Also, transferrin uptake was greatly reduced in cells expressing high levels of the inactive (but not the active) type IIβ enzyme but was also reduced in cells expressing either the active or inactive type IIα form. More studies are needed to define the exact steps in the endocytic pathway at which these enzymes may play a regulatory role.

None of the cells used in the present study display regulated secretion, a process in which PI 4-kinases have repeatedly been implicated. Therefore, it is quite possible that type II PI 4-kinases have an important function(s) in the secretory process or in any other more specialized membrane trafficking events, such as synaptic vesicle biogenesis (33). However, the wide tissue distribution of these enzymes and their presence in tissues and cells that lack regulated secretion suggest that they are involved in more basic processes of membrane dynamics. It will also be of great interest to follow the function of these proteins in membrane rafts because type II PI kinase activities have been shown to be present in such membrane subdomains (34). Given the pleiotropic functions of several members of

other inositide kinases, it is most likely that the type II enzymes are involved in multiple membrane fusion/budding events within mammalian cells.

The tissue distribution of the two enzymes does not indicate a specialized expression pattern for the individual proteins, which are probably both present simultaneously in numerous tissues and cells. The sizes of the main transcript for both proteins were 3.8–4.0 kb, in contrast to the 6.6-kb transcript size reported for PI4K type IIα (21). Because the tissue distribution for the latter transcript was found to be identical to that reported in Minogue *et al.* (21), we assume that the molecular size marker was misidentified in the latter report.

In a recent study, palmitoylation of PI4K type IIα has been shown to determine the membrane association of the protein (20). Although the palmitoylation motif of CCPCC (residues 170–174) is also present in PI4K type IIβ, the latter protein did not associate with early endosomes when lacking the N-terminal 96 amino acids. The presence of several proline residues within this part of the sequence of the type IIβ enzyme, including a PLLP motif, may be important in the localization of the protein. However, it is possible that palmitoylation is also required for proper membrane targeting.

In summary, the present study describes and characterizes a novel member of the type II PI 4-kinase family and compares its enzymatic characteristics to the recently cloned type IIα enzyme. It also demonstrates that, at least in COS-7 and HEK 293 cells, these enzymes are present in early endosomes through which both nutrient receptors and G protein-coupled receptors are processed during endocytosis. Expression of the more active type IIα enzyme also alters the distribution of membranes between the early and recycling endosomes and inhibits the rate of endocytosis of transferrin. These data suggest that this novel family of proteins is yet another addition to the increasing number of enzymes that regulate vesicular trafficking by modifying the phosphorylation state of phosphoinositides.

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